Shortening genetic transformation procedure by using green fluorescent protein marker

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With the successful plant regeneration from *H. brasiliensis* PB260 somatic embryos (Lardet *et al.*, 1999, Canadian Journal of Botany 77(8): 1168-1177) an A*grobacterium tumefaciens*-mediated genetic transformation has been developed (Blanc *et a*l., 2006, PCR 24(12) 724-733) and has lead to genetically transformed plant expressing the *gusA* reporter gene driven either by a *CAMV 35S* or the *Hev2.1* promoters.

Present procedure for genetic transformation

As shown in **figure 1**, following the coculture with *A. tumefaciens*, two decontamination and three selecting steps are performed in order to allow the transformed cells to growth properly. After a GUS assay, the highly transgenic calli lines are amplified on DM-P^{100} before molecular analysis and plant production.

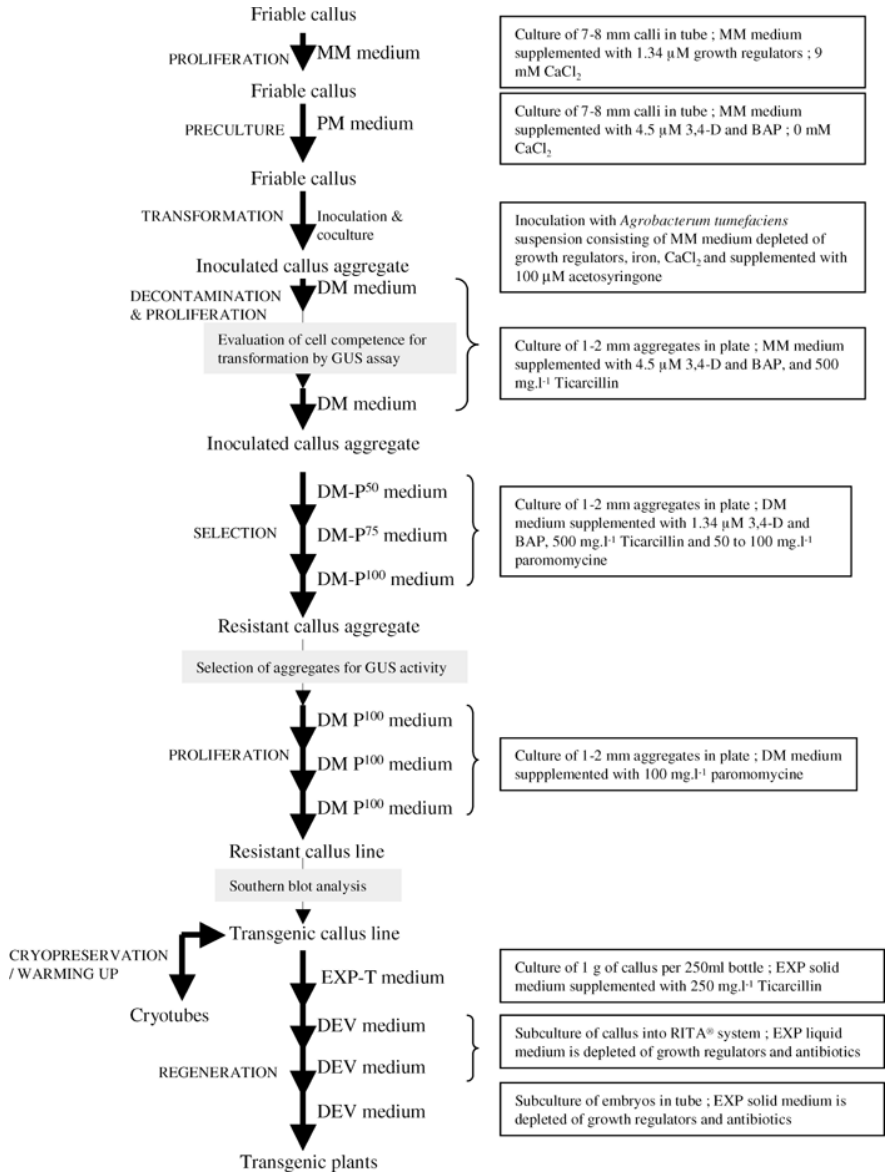

Figure 1: A*grobacterium tumefaciens*-mediated genetic transformation procedure (Blanc *et a*l., 2006).

Efficiency transformation evaluation

As the GUS assay is a visual but also destructive method, the use of a non-destructive visual reporter gene (GFP) would probably lead to a quicker selection of transformed aggregates.
For this purpose, a binary vector pCAMBIA2301 has been made with the two reporter genes and the *nptII* gene, all genes driven by the *CAMV 35S* promoter (**figure 2**). The vector was transferred in *A. tumefaciens* EHA105 strain.

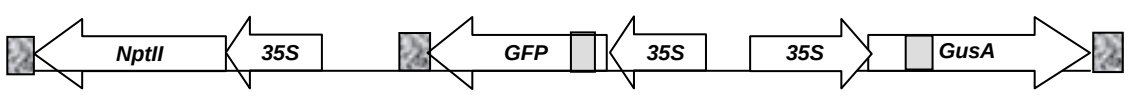

Figure 2: T-DNA of the binary vector pCAMBIA 2301-GFP containing a *gus* gene containing an intron and a *GFP* gene containing an intron as reporter genes and the *NptII* gene conferring paromomycine resistance.

The PB260 callus line CI05519 was cultivated 15 days before transformation on PM medium. After a 4 or 5-day coculture with EHA105-PCAMBIA2301-GFP, the cell competence for transformation was estimated either with the GUS assay or by using the GFP fluorescence analysis. (**Figure3**).

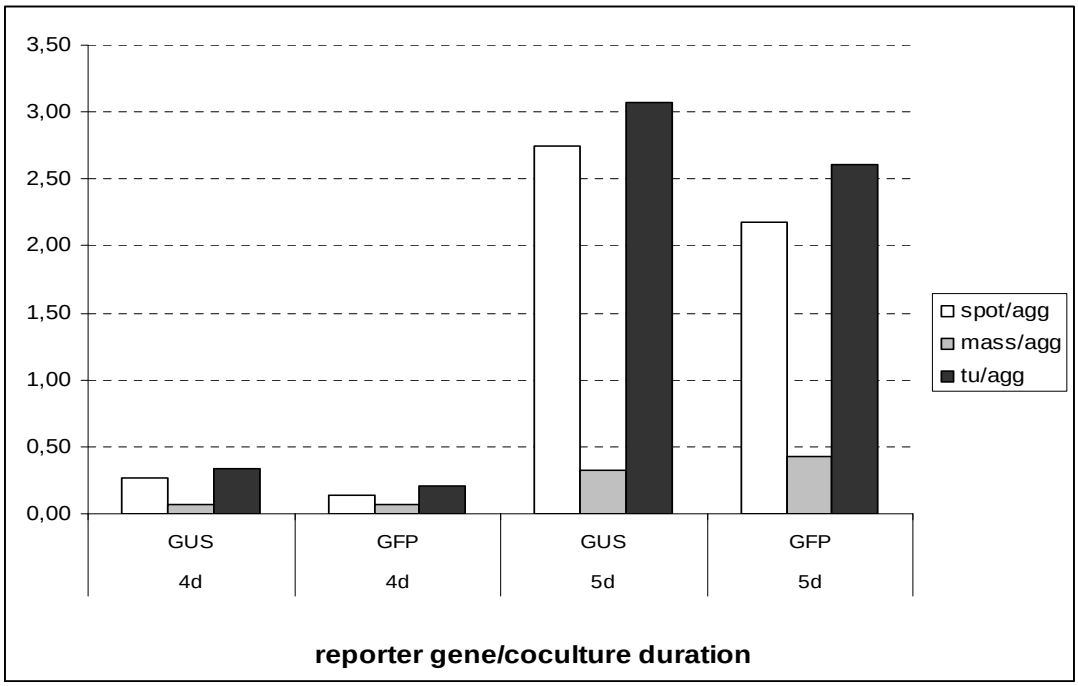

Figure 3: Evaluation of cell competence for transformation by using the GUS assay or the GFP fluorescence. The parameters measured are the numbers of spot per aggregate (spot/agg), number of mass per aggregate (mass/agg), and the total transformation units per aggregate (tu/agg).

Comparison of the GUS and GFP activities revealed that the two markers can be used to evaluate transformation efficiency as the same tendency was observed. In this experiment, a 5-day coculture gave the best results in terms of number of transformation units per aggregates and no obvious discrepancy was observed between the two preculture media.

Selection of transgenic callus lines

A total of 50 aggregates were kept for a procedure using only the GUS assay. For the procedure where only the GFP expressing aggregates were subcultured, a total of 24 aggregates were chosen.

As shown in figure 4, the green fluorescence was strongly visible in *H. brasiliensis* calli and that earlier selection of transgenic calli was thus possible from the first sub-culture on DM medium.

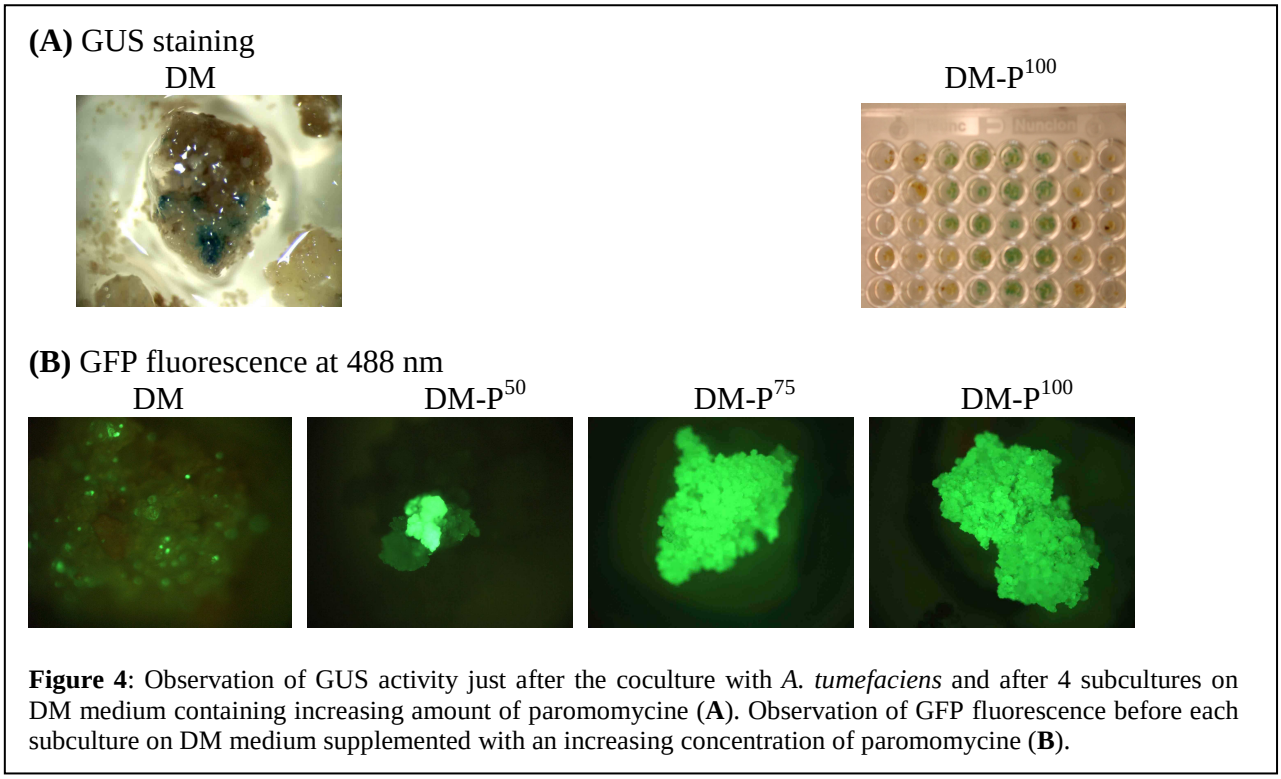

Figure 4: Observation of GUS activity just after the coculture with *A. tumefaciens* and after 4 subcultures on DM medium containing increasing amount of paromomycine (**A**). Observation of GFP fluorescence before each subculture on DM medium supplemented with an increasing concentration of paromomycine (**B**).

Advantage of the selection using the GFP activity

During the selection step, the number of aggregate was read and totalized. A preliminary calculation of the total number of sub cultured aggregates (**Table 1**) shows that the use of the GFP reduces drastically the number of sub cultured aggregates and hence it is a less time consuming procedure. Moreover, the preliminary results shows that the GFP selection is successful way to isolate transgenic calli lines, which is represented by the ratio of transgenic aggregates obtained per chosen aggregate.

Table 1: Number of sub cultured aggregates and transgenic calli lines established with using either GUS or GFP reporter gene.

	Reporter genes	
	GUS	**GFP**
Nb of aggregate for selection	25	12
Nb of aggregates on DM-P^{50}	>1000	>500
Nb of aggregates DM-P^{75}	>5000	<500
Aggregates DM-P^{100}	>2000	<300
Gus assay	Yes	No
Calli transgenic lines already under proliferation	2	6
Ratio transgenic agg/ total agg under selection (%)	8%	50%

A gain of time was also clearly observed as the selection of fully fluorescent calli was possible earlier.

Perspectives:

Hopefully, the GFP selection would allow the avoidance of antibiotic selection step and the use of binary vector containing no antibiotic resistance gene. All those tools will be applied for generating genetically transformed plant either over-expressing or silenced for candidate genes. Besides, GFP gene can be fused to genes of interest. Both transcriptional and translational fusions are useful approaches to follow the expression of genes driven by their own promoter through the GFP activity and the subcellular localization to have a better understanding of gene function in rubber tree cells.

Acknowledgements

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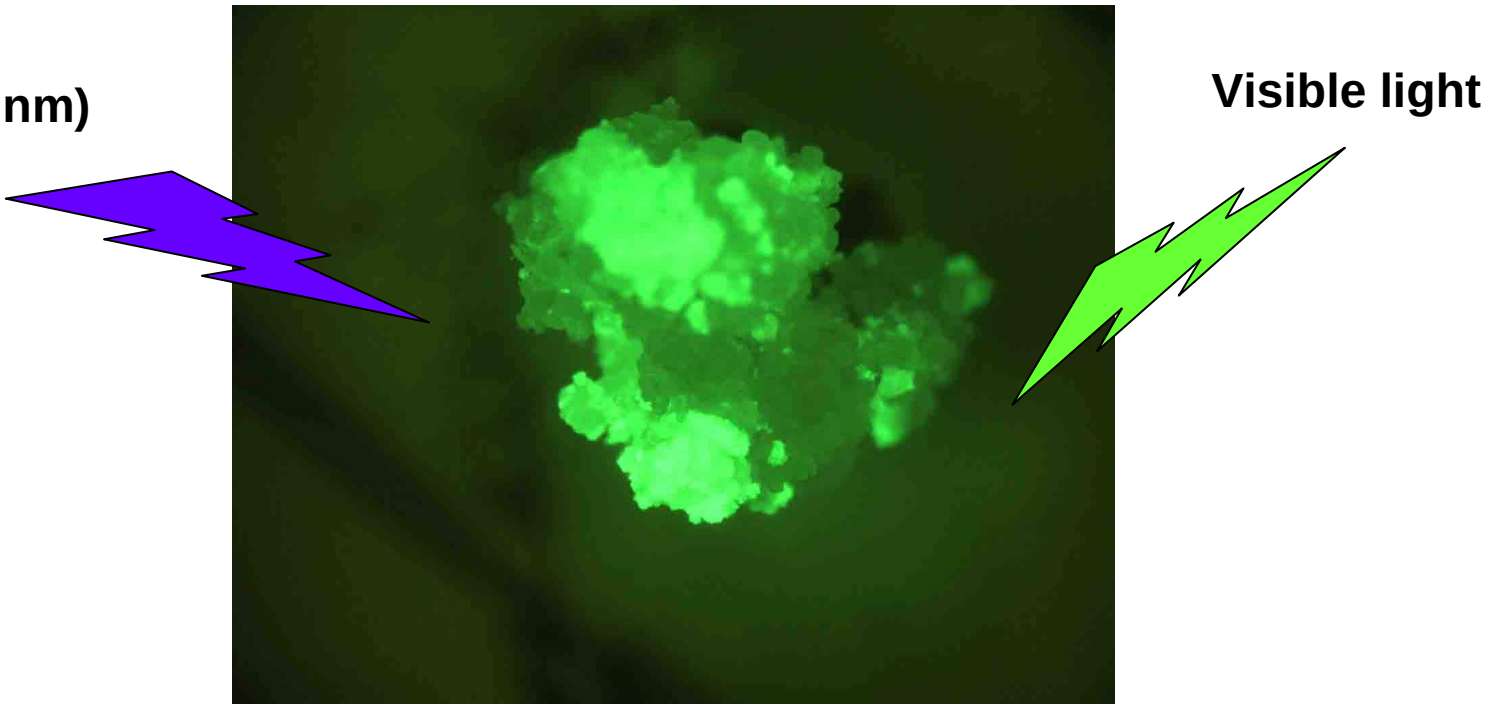
Shortening genetic transformation procedure by using green fluorescent protein marker



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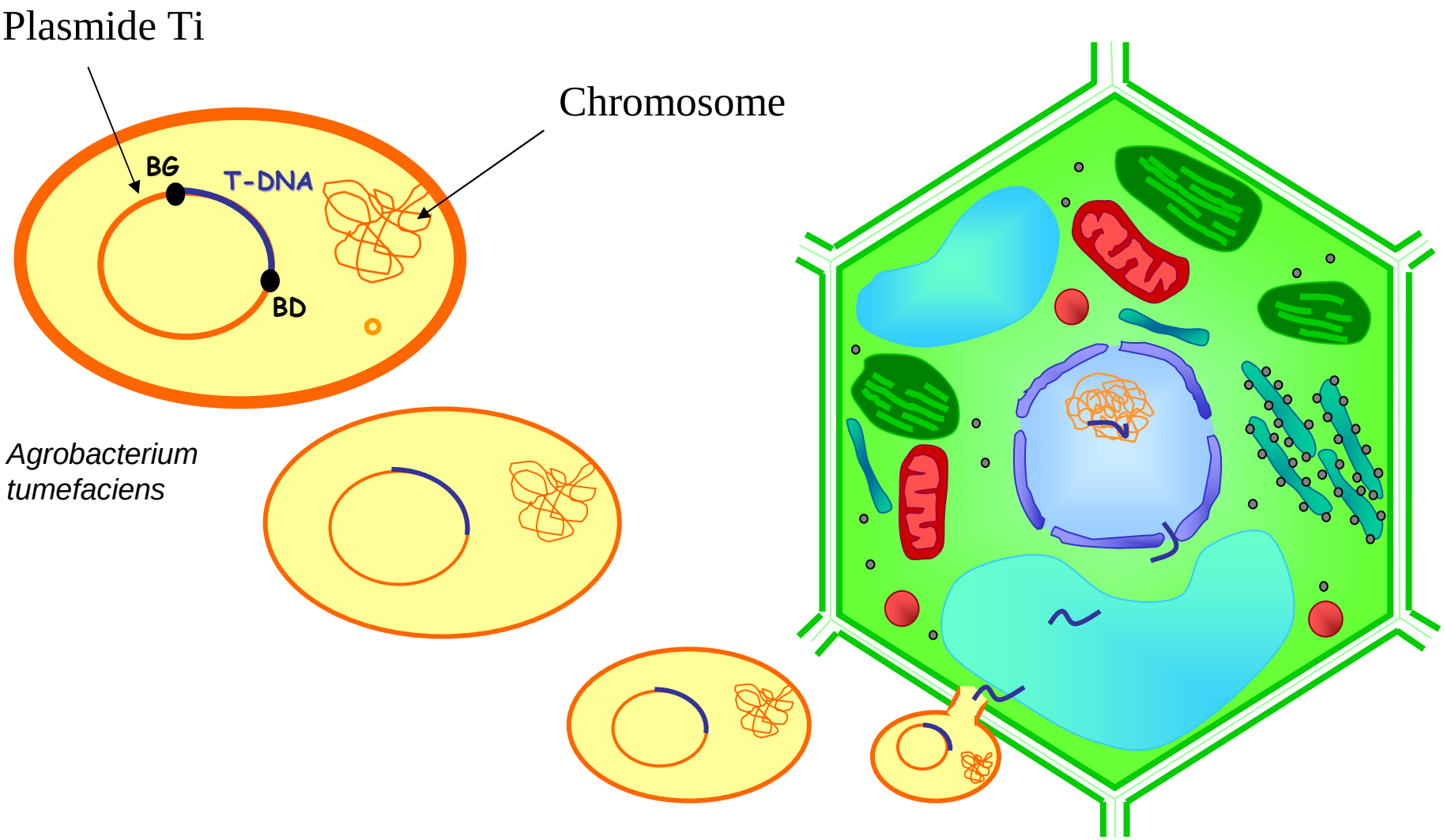
Genetic transformation

Stable integration of the T-DNA in plant genome



from J-C Breitler, CIRAD

Why using the GFP reporter gene?

- **GUS assay is a visual but also destructive method**
- **GFP is a non-destructive visual reporter gene**
  - **quicker selection of geneticcaly modified calli lines?**
  - **Avoid selection steps with antibiotics?**

=> Comparison and efficiency transformation evaluation (GUS and GFP)

- **Binary vector containing 3 genes under the CAMV35 promotor :**
  - *GusA*
  - *GFP*
  - *NPTII*

pCAMBIA2301

H. Brasiliensis genetic modification procedure

Cryopreserved PB 260 calli

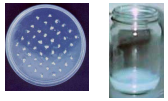
CI05519

Friable callus

PRECULTURE

co-cultivated friable callus

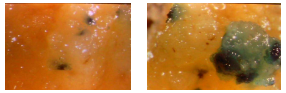
COCULTURE



Inoculation
A. tumefaciens

DECONTAMINATION (Ticarcillin)

Tissus proliferation



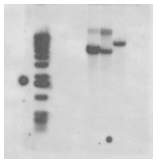
SELECTION (Paromomycine)

Resistant tissus

PROLIFERATION

Resistant calli lines

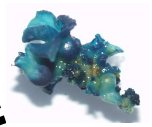
Molecular analysis



Transgenic calli lines

REGENERATION

Transgenic embryos



GMO plants

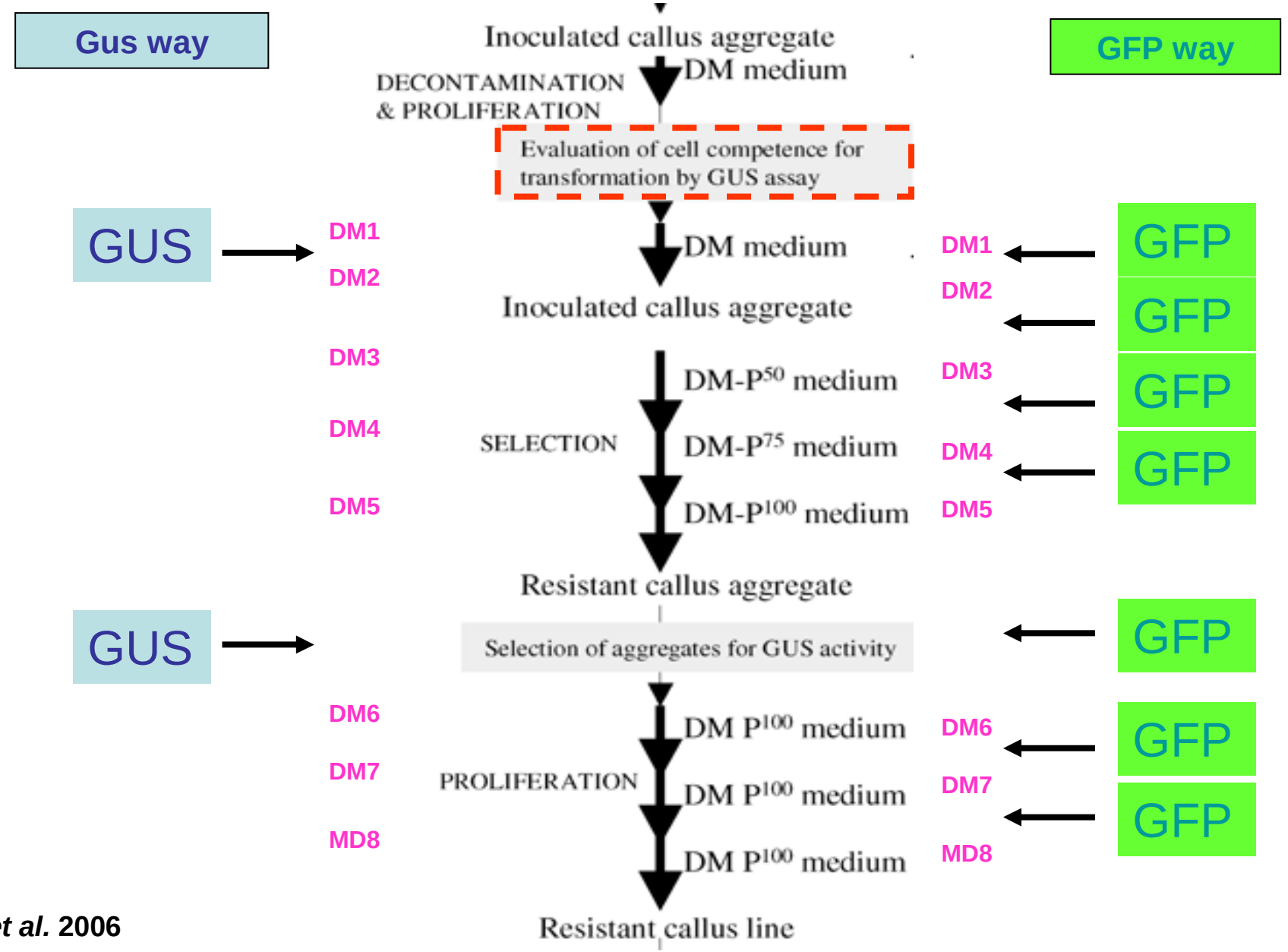
Lardet et *al*., 1999

Montoro *et al.* 2000, 2003

1998-2002
Genetic modification of PB 260 friable calli

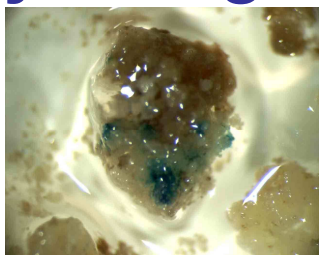
2002-2004
Obtention of GMO plants *35S::GUS* (11 lines) and *H4::GUS* (16 lines)

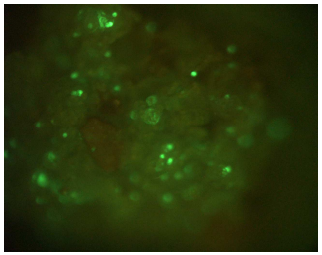
Focus on decontamination, selection and proliferation steps

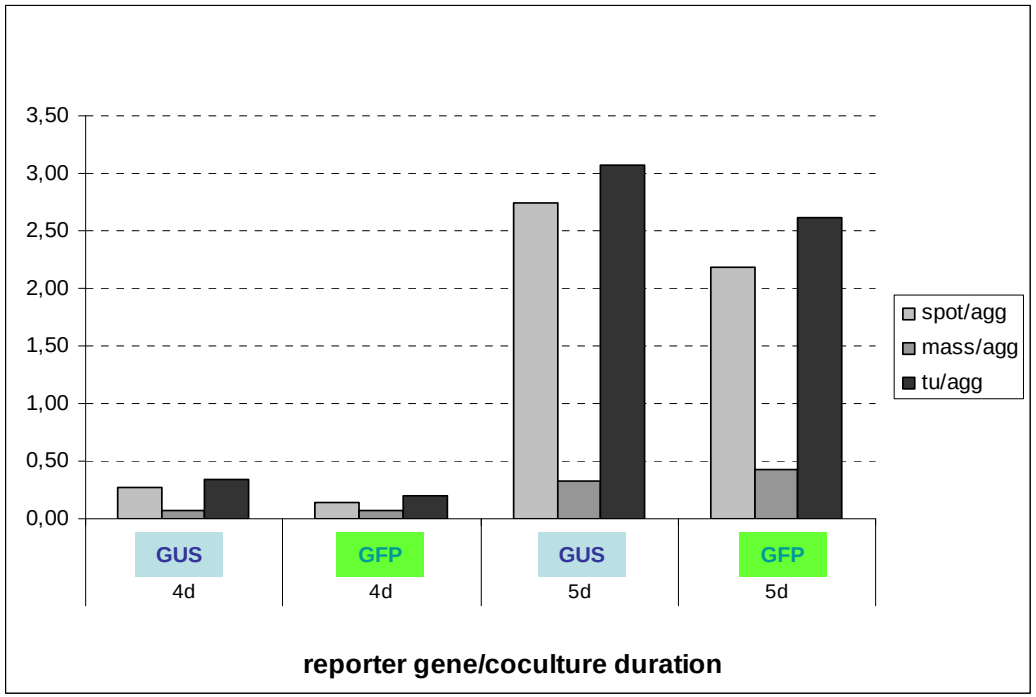


Blanc *et al.* 2006

Evaluation of cell competence for transformation by using the GUS assay or the GFP fluorescence





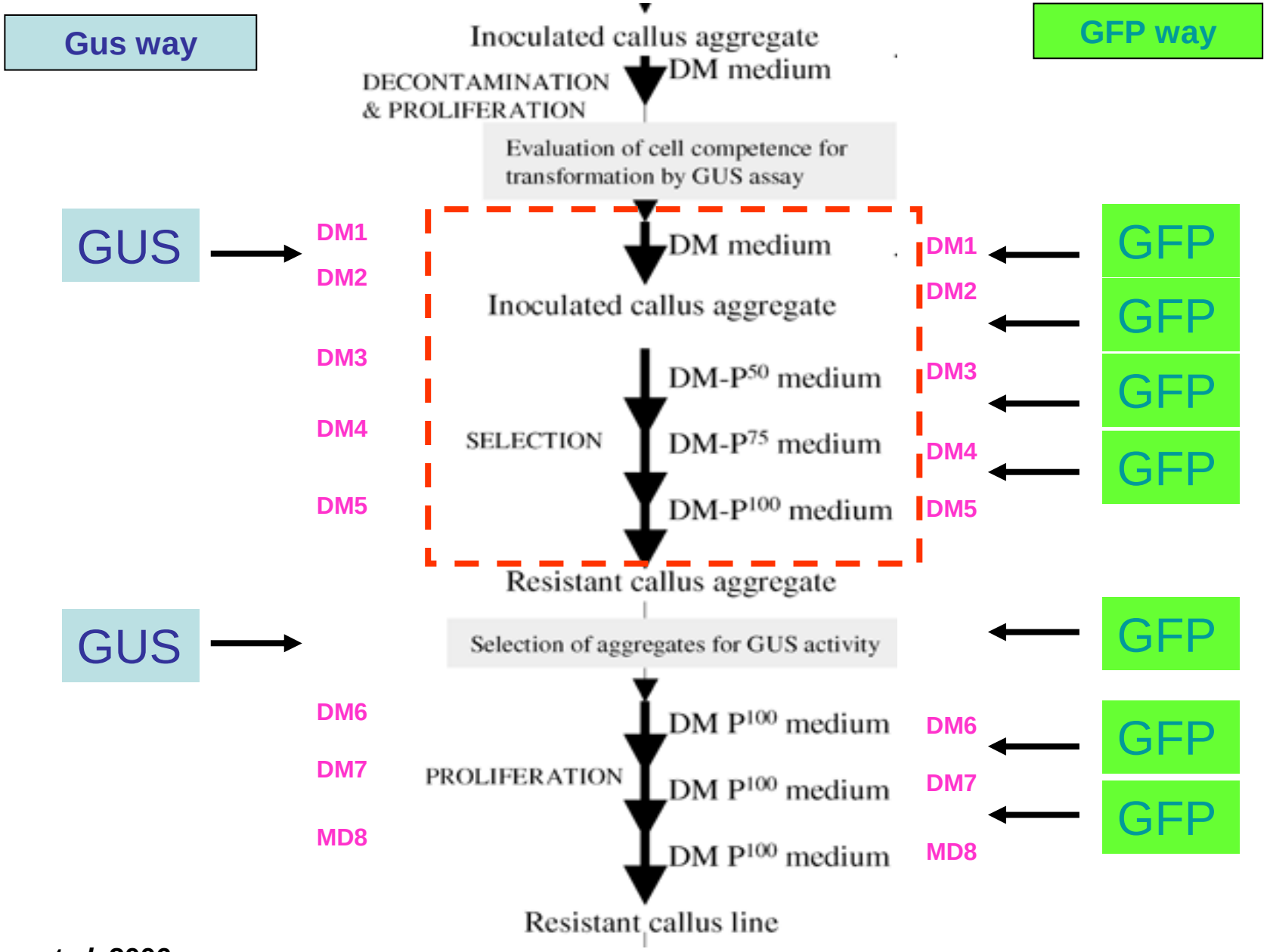


The parameters measured are:
- number of spot per aggregate (spot/agg),
- number of mass per aggregate (mass/agg),
- total transformation units per aggregate (tu/agg)

Comparison of the GUS and GFP activities revealed that:

=> The two markers can be used to evaluate transformation efficiency as the same tendency was observed
=> In this experiment, a 5-day coculture gave the best results in terms of number of transformation units per aggregates

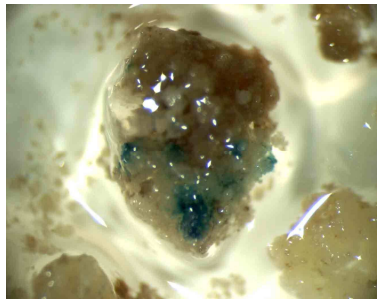
Focus on selection and proliferation steps



Blanc *et al.* 2006

Focus on selection and proliferation steps

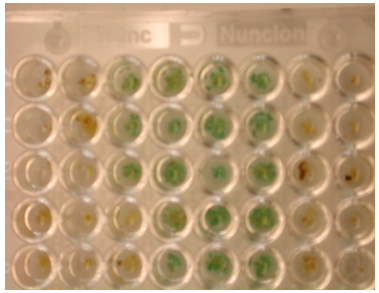
DM



Gus way

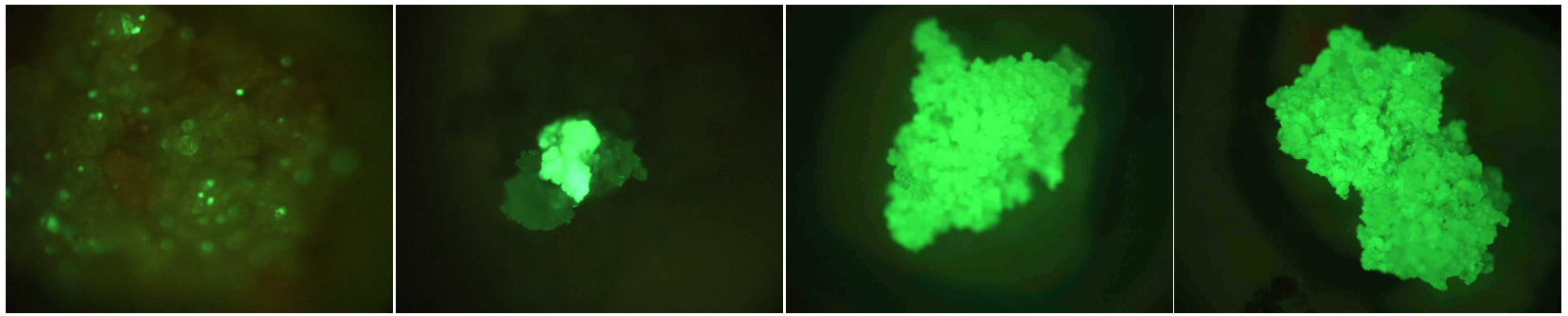
Time consuming work

DM5



GFP way

DM1 **DM3** **DM4** **DM5**



=> Some of the transgenic calli are already fully fluorescent on MD4

Advantage of the selection using the GFP fluorescence

Number of sub cultured aggregates and transgenic calli lines established with using either GUS or GFP reporter gene

	Reporter genes	
	GUS	**GFP**
Nb of aggregate for selection	50	24
Nb of aggregates on DM3	>1000	>500
Nb of aggregates DM4	>5000	<500
Aggregates DM5	>2000	<300
Gus assay	Yes	No
Calli transgenic lines already under proliferation	3	12
Ratio transgenic agg/ total agg under selection (%)	**6 %**	**50 %**

=> The use of the GFP reduces drastically the number of sub cultured aggregates and hence it is a less time consuming procedure

=> Preliminary results shows that the GFP selection is successful way to isolate transgenic calli lines, which is represented by the ratio of transgenic aggregates obtained per chosen aggregate.

Conclusions - Perspectives

- Shortening genetic transformation procedure by using green fluorescent protein marker (MD1-MD3-MD5)
- Actually, generation of genetically transformed plant over-expressing candidate genes
- the GFP visual selection would allow the avoidance of antibiotic selection step and the use of binary vector containing no antibiotic resistance gene.
- GFP gene can be fused to genes of interest

=> Both transcriptional and translational fusions are useful approaches to follow the expression of genes driven by their own promoter through the GFP activity and the subcellular localization to have a better understanding of gene function in rubber tree cells.

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